PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 14 May 1998 (14.05.98)	
International application No. PCT/US97/15611	**Applicant's or agent's file reference** 971177
International filing date (day/month/year) 05 September 1997 (05.09.97)	**Priority date** (day/month/year) 06 September 1996 (06.09.96)
Applicant BRONSHTEIN, Victor	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

03 April 1998 (03.04.98)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer A. Karkachi
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15611

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A01N 1/02
US CL :435/1.1, 1.2, 1.3; 435/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/1.1, 1.2, 1.3; 435/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 4,980,277 A (JUNNILA) 25 December 1990, column 3, lines 58-69.	1-8, 10, 11, 16, 18-24 ---------- 9, 12-15, 17
X — Y	TITTERINGTON et al. Synthetic and biological macromolecules: protection of mouse embryos during cryopreservation by vitrification. Human Reproduction. 1995, Vol. 10, No. 3, pages 649-653, especially Table III.	1-7, 16, 18-24 ---------- 8-15, 17
X — Y	RALL et al. High *in vitro* and *in vivo* survival of day 3 mouse embryos vitrified or frozen in a non-toxic solution of glycerol and albumin. Journal of Reproduction and Fertility. 1994, Vol. 101, pages 681-688, especially pages 682-683.	1-6, 9, 10, 16, 18-24 ---------- 7, 8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 DECEMBER 1997	26 JAN 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SANDRA SAUCIER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15611

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,160,313 A (CARPENTER et al.) 03 November 1992, especially columns 3 and 4.	1-7, 10-16, 18-24 --- 8, 9
X --- Y	US 5,217,860 A (FAHY et al.) 08 June 1993, especially column 25.	1, 6, 8, 22-24 --- 9-16, 22, 23
X --- Y	US 5,364,756 A (LIVESEY et al.) 15 November 1994, especially examples.	1-7, 9, 11, 12, 14-23 --- 8, 10, 13

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 971177	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US97/15611	International filing date *(day/month/year)* 05 SEPTEMBER 1997	Priority date *(day/month/year)* 06 SEPTEMBER 1996
International Patent Classification (IPC) or national classification and IPC IPC(6): A01N 1/02 and US Cl.: 435/1.1, 1.2, 1.3, 435/2		
Applicant UNIVERSAL PRESERVATION TECHNOLOGIES, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of ___ sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
03 APRIL 1998	03 DECEMBER 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SANDRA SAUCIER Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (January 1994)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/15611

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

[X] the international application as originally filed.

[X] the description, pages 1-18 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.

[X] the claims, Nos. 1-24 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ none , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages none .
[X] the claims, Nos. none .
[X] the drawings, sheets/~~fig~~ none .

3. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/15611

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	17	YES
	Claims	1-16, 18-24	NO
Inventive Step (IS)	Claims	none	YES
	Claims	1-24	NO
Industrial Applicability (IA)	Claims	1-24	YES
	Claims	none	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-8, 10, 16, 18-24 lack novelty under PCT Article 33(2) as being anticipated by US 4,980,277 to Junnila.

Claims 1-17 are directed to a method of preserving cells or tissue by contacting the cell or tissue with a solution comprising 1) a non-permeating co-solute selected from amino acids, betaine, sugar alcohol, monosaccharides, ketose monosaccharides, amino sugars, alditol, inositol, aidonic, uronic, aldaric acids, disaccharides and polysaccharides, 2) a permeating cryoprotectant comprising dimethylsulfoxide, ethylene glycol, propylene glycol and glycerol, and 3) a non-permeating cryoprotectant comprising dextran, starch, polyethylene glycol, PVP, Ficol and peptides. Also, there appear to be some claims directed to rehydration of the tissue or cells. Compositions used in the method are also claimed.

US 4,980,277 discloses a method of cryopreserving cells comprising treating the cells with a composition of 1) betaine in a concentration of 1-30%, 2)glycerol and 3) egg yolk which contains peptides.

Claims 1-7, 16, 18-24 lack novelty under PCT Article 33(2) as being anticipated by Titterington et al.

Titterington et al. disclose a method of cryopreservation of mouse embryos comprising treating the embryos with a composition containing 1)sucrose 0.75 M, 2) glycerol, and 3) Percoll.

Claims 1-6, 9, 10, 16, 18-24 lack novelty under PCT Article 33(2) as being anticipated by Rall et al.

Rall et al. disclose a method of cryopreserving embryos comprising treating the embryos with a composition comprising 1) glucose, 2)glycerol 6.5M, 3) BSA, which contains albumin (see page 683, Vitrification solutions). The concentration of the components of the solution is increased stepwise (see page 682, Exposure of embryos to the vitrification solution).

(Continued on Supplemental Sheet.)

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 11-15 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s): No antecedent basis exists for the recitation of "wherein the rehydration solution further comprises a permeating rehydration cryoprotectant". No rehydration solution is mentioned in claims 1 or 2.

Claim 4 should recite "permeating" cryoprotectant.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
Claims 1-6, 10, 16, 18-24 lack novelty under PCT Article 33(2) as being anticipated by US 5,160,313 to Carpenter et al.

US 5,160,313 discloses a method of cryopreserving tissue comprising contacting the tissue with a composition comprising 1)DMEM which contains glucose, 2) DMSO, 3) fetal calf serum (example 1).

Claims 1, 6, 8-10, 22-24 lack novelty under PCT Article 33(2) as being anticipated by US 5,217,860 to Fahy et al.

US 5,217,860 disclose a method of cryopreserving tissue comprising adding a solution containing 1)formamide and 2) DMSO, increasing the concentration of the components of the solution according to a desired profile.

Claims 1-7, 9, 11-16, 18-23 lack novelty under PCT Article 33(2) as being anticipated by US 5,364,756 to Livesey et al.

US 5,364,756 disclose a method of preserving cells (column 4, line 60) by contacting the cells with a solution containing 1) raffinose,2) DMSO and 3) dextran, see column 13, tables. The sample is then dried using the temperature program of column 17, lines 32-46. The sample is stored and then rehydrated with a solution containing 1)DMSO, 2)trehalose, 3) dextran for example, see column 20, table. The rehydration solution was then diluted by addition of cell culture medium.

Claims 1-24 lack an inventive step under PCT Article 33(3) as being obvious over US 5,364,756 in view of US 4,865,871 and US 5,217,860 or Rall et al.

The use of stepwise increase in the concentration of cryopreservatives prior to freezing in the place of the one step addition method as disclosed by US 5,364,756 would have been obvious when US 5,364,756 was taken with US 5,217,860 or Rall et al. which disclose stepwise increases in the concentration of cryoprotectants in order to decrease osmotic stress. The stability of the processed tissue as disclosed by US 5,364,756 is assumed to be the same as the claimed stability because the process which produces the stable product, as claimed, is essentially the same as the process disclosed in the prior art, particular see US 4,865,871 which discloses the method of sublimation and storage of the biological samples.

All of the component of the cryoprotective solution and the rehydration solution are the same as those used in the prior art. Thus, the invention as a whole lacks an inventive step.

Applicants argue that the prior art does not teach that the product can be stably stored at a temperature greater than 4°C. First, the claims do not have a storage step, claim 17 merely recites a desired result, it is not a method step. Second, even if the recitation were a method step, the time limit of storage is not in the claim, thus the time of "stable storage" may be for any length of time, even very brief ones lasting an hour or less. Third, neither the degree of stability, nor the end point for a test of stability is recited in the claim or disclosed. Thus, "stably" may mean a variety of degrees stability. For example, in the blood banking field, a red cell concentrate may be used for transfusion after two weeks of storage, although it may show signs of hemolysis and decreased ATP levels and other biochemical parameters. This concentrate may be considered to be a stably stored preparation for the purposes of transfusion, but not to be a stably stored preparation when the test is a biochemical one.

Further, US 5,364,756 discloses the preservation and storage of erythrocytes treated with glycerol and dextran, see Example 4. The drying method is stated to be described in US 4,865,871. US 4,865,871 teach the application of heat so that the temperature of a vitrified specimen is raised through (above) the glass transition temperature and reaches room temperature (col. 11, l. 1-20. Thus, the red cells of '756 are taught to be stored under vacuum at room temperature for up to two weeks. Thus, applicant's arguments are unpersuasive in view of both the disclosure, claims and the prior art.

---------- NEW CITATIONS ----------
US 4,865,871 A (LIVESEY ET AL.) 12 September 1989, see column 11, lines 1-18.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: BARBARA E. JOHNSON
WEBB ZIESENHEIM BRUENING LOGSDON ORKIN & HANSON , P.C.
700 KOPPERS BUILDING, 436 SEVENTH AVENUE
PITTSBURGH, PA 15219-1818

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	11 JAN 1999

Applicant's or agent's file reference	IMPORTANT NOTIFICATION
971177	

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US97/15611	05 SEPTEMBER 1997	06 SEPTEMBER 1996

Applicant
UNIVERSAL PRESERVATION TECHNOLOGIES, INC.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SANDRA SAUCIER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

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PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: BARBARA E. JOHNSON
WEBB ZIESENHEIM BRUENING LOGSDON ORKIN & HANSON , P.C.
700 KOPPERS BUILDING, 436 SEVENTH AVENUE
PITTSBURGH, PA 15219-1818

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	11 JAN 1999

Applicant's or agent's file reference	IMPORTANT NOTIFICATION
971177	

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US97/15611	05 SEPTEMBER 1997	06 SEPTEMBER 1996

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Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)★

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 971177	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US97/15611	International filing date *(day/month/year)* 05 SEPTEMBER 1997	Priority date *(day/month/year)* 06 SEPTEMBER 1996
International Patent Classification (IPC) or national classification and IPC IPC(6): A01N 1/02 and US Cl.: 435/1.1, 1.2, 1.3, 435/2		
Applicant UNIVERSAL PRESERVATION TECHNOLOGIES, INC.		

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3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
03 APRIL 1998	03 DECEMBER 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SANDRA SAUCIER Telephone No. (703) 308-0196

Form PCT/IPEA/409 (cover sheet) (January 1994)★

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/15611

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

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pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.

[X] the claims, Nos. 1-24 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

[X] the drawings, sheets/~~fig~~ none , as originally filed.
sheets/~~fig~~ NONE , filed with the demand.
sheets/~~fig~~ NONE , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

[X] the description, pages none.

[X] the claims, Nos. none.

[X] the drawings, sheets/~~fig~~ none.

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4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US97/15611

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	17	YES
	Claims	1-16, 18-24	NO
Inventive Step (IS)	Claims	none	YES
	Claims	1-24	NO
Industrial Applicability (IA)	Claims	1-24	YES
	Claims	none	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-8, 10, 16, 18-24 lack novelty under PCT Article 33(2) as being anticipated by US 4,980,277 to Junnila.

Claims 1-17 are directed to a method of preserving cells or tissue by contacting the cell or tissue with a solution comprising 1) a non-permeating co-solute selected from amino acids, betaine, sugar alcohol, monosaccharides, ketose monosaccharides, amino sugars, alditol, inositol, aidonic, uronic, aldaric acids, disaccharides and polysaccharides, 2) a permeating cryoprotectant comprising dimethylsulfoxide, ethylene glycol, propylene glycol and glycerol, and 3) a non-permeating cryoprotectant comprising dextran, starch, polyethylene glycol, PVP, Ficol and peptides. Also, there appear to be some claims directed to rehydration of the tissue or cells. Compositions used in the method are also claimed.

US 4,980,277 discloses a method of cryopreserving cells comprising treating the cells with a composition of 1) betaine in a concentration of 1-30%, 2)glycerol and 3) egg yolk which contains peptides.

Claims 1-7, 16, 18-24 lack novelty under PCT Article 33(2) as being anticipated by Titterington et al.

Titterington et al. disclose a method of cryopreservation of mouse embryos comprising treating the embryos with a composition containing 1)sucrose 0.75 M, 2) glycerol, and 3) Percoll.

Claims 1-6, 9, 10, 16, 18-24 lack novelty under PCT Article 33(2) as being anticipated by Rall et al.

Rall et al. disclose a method of cryopreserving embryos comprising treating the embryos with a composition comprising 1) glucose, 2)glycerol 6.5M, 3) BSA, which contains albumin (see page 683, Vitrification solutions). The concentration of the components of the solution is increased stepwise (see page 682, Exposure of embryos to the vitrification solution).

(Continued on Supplemental Sheet.)

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 11-15 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s): No antecedent basis exists for the recitation of "wherein the rehydration solution further comprises a permeating rehydration cryoprotectant". No rehydration solution is mentioned in claims 1 or 2.

Claim 4 should recite "permeating" cryoprotectant.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
Claims 1-6, 10, 16, 18-24 lack novelty under PCT Article 33(2) as being anticipated by US 5,160,313 to Carpenter et al.

US 5,160,313 discloses a method of cryopreserving tissue comprising contacting the tissue with a composition comprising 1)DMEM which contains glucose, 2) DMSO, 3) fetal calf serum (example 1).

Claims 1, 6, 8-10, 22-24 lack novelty under PCT Article 33(2) as being anticipated by US 5,217,860 to Fahy et al.

US 5,217,860 disclose a method of cryopreserving tissue comprising adding a solution containing 1)formamide and 2) DMSO, increasing the concentration of the components of the solution according to a desired profile.

Claims 1-7, 9, 11-16, 18-23 lack novelty under PCT Article 33(2) as being anticipated by US 5,364,756 to Livesey et al.

US 5,364,756 disclose a method of preserving cells (column 4, line 60) by contacting the cells with a solution containing 1) raffinose,2) DMSO and 3) dextran, see column 13, tables. The sample is then dried using the temperature program of column 17, lines 32-46. The sample is stored and then rehydrated with a solution containing 1)DMSO, 2)trehalose, 3) dextran for example, see column 20, table. The rehydration solution was then diluted by addition of cell culture medium.

Claims 1-24 lack an inventive step under PCT Article 33(3) as being obvious over US 5,364,756 in view of US 4,865,871 and US 5,217,860 or Rall et al.

The use of stepwise increase in the concentration of cryopreservatives prior to freezing in the place of the one step addition method as disclosed by US 5,364,756 would have been obvious when US 5,364,756 was taken with US 5,217,860 or Rall et al. which disclose stepwise increases in the concentration of cryoprotectants in order to decrease osmotic stress. The stability of the processed tissue as disclosed by US 5,364,756 is assumed to be the same as the claimed stability because the process which produces the stable product, as claimed, is essentially the same as the process disclosed in the prior art, particular see US 4,865,871 which discloses the method of sublimation and storage of the biological samples.

All of the component of the cryoprotective solution and the rehydration solution are the same as those used in the prior art. Thus, the invention as a whole lacks an inventive step.

Applicants argue that the prior art does not teach that the product can be stably stored at a temperature greater than 4°C. First, the claims do not have a storage step, claim 17 merely recites a desired result, it is not a method step. Second, even if the recitation were a method step, the time limit of storage is not in the claim, thus the time of "stable storage" may be for any length of time, even very brief ones lasting an hour or less. Third, neither the degree of stability, nor the end point for a test of stability is recited in the claim or disclosed. Thus, "stably" may mean a variety of degrees stability. For example, in the blood banking field, a red cell concentrate may be used for transfusion after two weeks of storage, although it may show signs of hemolysis and decreased ATP levels and other biochemical parameters. This concentrate may be considered to be a stably stored preparation for the purposes of transfusion, but not to be a stably stored preparation when the test is a biochemical one.

Further, US 5,364,756 discloses the preservation and storage of erythrocytes treated with glycerol and dextran, see Example 4. The drying method is stated to be described in US 4,865,871. US 4,865,871 teach the application of heat so that the temperature of a vitrified specimen is raised through (above) the glass transition temperature and reaches room temperature (col. 11, l. 1-20. Thus, the red cells of '756 are taught to be stored under vacuum at room temperature for up to two weeks. Thus, applicant's arguments are unpersuasive in view of both the disclosure, claims and the prior art.

NEW CITATIONS

US 4,865,871 A (LIVESEY ET AL.) 12 September 1989, see column 11, lines 1-18.

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(57) Abstract

The method of preservation by vitrification, described in the present application, provides for storage of samples at higher temperatures than in conventional methods and can be applied to cells, multicellular tissues, organs and organismes. The method of the present invention includes preparing a solution of vitrification non-permeating co-solutes (amino acids, betaines, carbohydrates, or other non-permeating co-solutes that effectively decrease the chemical potential of permeating cryoprotectants in aqueous solutions), a permeating cryoprotectant and a non-permeating cryoprotectant (polyvinylpyrrolidone, polyethylene glycol, dextran, hydroxy ethyl starch, Ficol, etc.), contacting a sample with the vitrification solution and storing the sample at a storage temperature. The method also includes the step of rehydrating the preserved sample in a rehydration solution prepared in the manner of the vitrification storage solution. The present invention is also directed to a vitrification solution and a rehydration solution as described in connection with the method.

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VITRIFICATION SOLUTIONS FOR SHELF PRESERVATION OF CELLS, TISSUES, ORGANS AND ORGANISMS

BACKGROUND OF THE INVENTION

1. Field of Invention

This invention relates to the long-term shelf preservation of cells and multicellular specimens by vitrification. The invention is directed to the optimization of vitrification and rehydration solutions, as well as vitrification, and rehydration procedures.

2. Description of the Related Art

Low temperature preservation of cells and multicellular specimens by traditional freezing methods is not uncommon. However, the strong damaging action of ice crystallization limits the effectiveness of such cryogenic methods to the cryopreservation of single cells and multicellular specimens. Vitrification is an alternative approach to cryopreservation that utilizes solidification of samples during cooling, without formation of ice crystals (Fahy, G.M. et al., 1984). Conventionally, cryopreservation by vitrification of single cell (erythocyte, stem cells, sperm, *E. Coli*, yeasts and other cellular microorganisms, etc.) and multicellular specimens provide for storage of cryopreserved samples at -196°C in liquid N_2. However, there is currently a need for reliable methods for long-term shelf preservation at refrigeration or higher temperatures. We believe that development of these methods was not possible because of several generally accepted misconceptions and deficiencies of the prior art that have been addressed by the inventor (Bronshtein, V.L., 1995a).

Effects of dehydration

Ice formation at low temperatures can be avoided only if samples are sufficiently dehydrated. Dehydration is known to damage cells. The damaging effect of dehydration increases with increasing osmotic pressure (concentration) and depends strongly upon whether the vitrification solution contains permeating cryoprotectants. For example, cells normally cannot survive equilibration in

solutions containing only non-permeating solutes in concentration >1 mol/l. However, many types of cells can easily tolerate equilibration in solutions containing permeating cryoprotectants in much higher concentrations. This is because penetration of cryoprotectants protects cells against dehydration damage.

Here, it is important to note that dehydration does not mean a decrease in the cell volume which actually may be very damaging (Meryman, H.T., 1967, Meryman, H.T., 1970). The term "dehydration" means removal of water, or increase in the osmotic pressure. Erroneous use of this term resulted in several misconceptions. For example, as described below, dehydration by itself is not a strong damaging factor. Dehydration may even be a protective factor, as performed according to the present invention.

As shown in Bryant, G. et al. (1992) damage of unloaded specimens during dehydration in vitrification solution is caused by hydration forces occurring between biological macromolecules and membranes when distances between them become small as a result of dehydration. It is believed that loading of cells with permeating cryoprotectants, protects against subsequent dehydration because intracellular cryoprotectant diminishes these forces. Therefore, some amount of intracellular cryoprotectants are required to protect cells during dehydration to high osmotic pressures. For this reason, Rall proposed equilibration of biological specimens in loading solutions of permeating cryoprotectants (dimethylsulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), glycerol, etc.) prior to dehydration, in order to reduce the strong damaging effect of dehydration in the vitrification solution (Rall, W.F. et al., 1985a). Unfortunately, the protective effect of loading significantly decreases with increasing time of equilibration in vitrification solution. Currently, this effect is erroneously explained as a direct toxic effect of high concentration of intracellular cryoprotectants.

Apparent toxicity of vitrification solution

Based on the general belief that intracellular cryoprotectants help to vitrify cytosol, and the fact that some intracellular cryoprotectant is required to protect cells during dehydration, penetration of cryoprotectant inside cells may be considered as a beneficial phenomena. A negative aspect of this penetration, considered in the literature, is associated with direct chemical toxicity of cryoprotectants (Fahy et al., 1990). Because the toxicity is believed to be proportional to the concentration of cryoprotectants (not to the amount of cryoprotectants inside a cell) three basic approaches have been proposed to minimize the toxicity (for details see review of Steponkus, P.L. et al., 1992):

1. to use a mixture of different cryoprotectants;

2. to add components that may act as "toxicity neutralizers"; and

3. to identify solutes that will form a glass at a lower concentration.

However, Fahy found that biochemical studies of the toxicity to date have not adequately demonstrated the mechanisms of toxicity (Fahy et al., 1990). This actually means that the direct chemical toxicity of typical permeating cryoprotectants (EG, PG, glycerol and DMSO) is small. Therefore, in agreement with the conclusion of Fahy et al., 1990, present concepts of cryoprotectant toxicity are in need of serious revision.

Recently, Langis, R. et al. (1990) demonstrated that survival of isolated rye protoplast, following a dehydration step, is a function of osmolarity rather than the concentration of vitrification solutions. Based on this observation, Steponkus, P.L. et al. (1992) discussed an alternative strategy for formulating less toxic solutions with lower osmolarity.

As mentioned above, cells can tolerate dehydration in very concentrated vitrification solution for several minutes if they have been loaded with permeating cryoprotectants. However, during long equilibration times in vitrification solution, cell survival decreases with increasing time of equilibration. Because loading of cells with permeating cryoprotectants protects against injury subsequently occurred after dehydration in vitrification solutions, in the case of short dehydration times one may suggest that the injury depends primarily on osmolarity. However, because the concentration of intracellular cryoprotectants that is reached after dehydration increases with increasing osmolarity of vitrification solution, the existing experimental observations do not answer the question whether damage of dehydrated embryos is a result of the increased concentration of intracellular cryoprotectant, or the increase in osmotic pressure. In both cases, however, the questions as to why the injury increases with dehydration time remains to be answered. It is also very important because the time required to complete dehydration of multicellular specimens can be substantially longer than that for individual cells.

Bronshteyn, V.L. et al. (1994) and Steponkus, P.L. et al. (1994) discussed an alterative strategy for formulating less toxic solutions with lower osmolarity. As mentioned above, cells can tolerate dehydration in very concentrated vitrification solution for several minutes if they have been loaded with permeating cryoprotectants. However, during longer equilibration times in vitrification solutions, cell survival decreases with increasing time of equilibration. Because loading of cells with permeating cryoprotectants protects against injury occurring after dehydration in vitrification solution, in the case of short dehydration times, one may suggest that the injury depends primarily on osmolarity. However, because the concentration of intracellular cryoprotectant that is reached after dehydration increases with increasing

osmolarity of vitrification solution, the existing experimental observations do not answer the question of whether damage is a result of the increased concentration of intracellular cryoprotectant or an increase in osmotic pressure. In both cases, no answer is presented as to why injury increases with dehydration time. This answer is very important because the time required to complete dehydration of multicellular specimens can be substantially longer than that for individual cells.

Bronshteyn, V.L. et al. (1994) and Steponkus, P.L. et al. (1994) suggest that a significant part of the apparent toxicity of ethylene glycol-based vitrification for loaded *Drosophila melanogaster* embryos is associated with ethylene glycol permeation (increase in mass of ethylene glycol inside embryos) rather than with chemical toxicity of intra-embryo ethylene glycol, or osmotic pressure of vitrification solution. The injurious effect of permeation of cryoprotectants during equilibration in vitrification solution was also demonstrated in the studies performed with mouse embryos (Zhu, S.E. et al., 1993, Tachikawa, S. et al., 1993 and Kasai, M. et al., 1990). This toxic effect is not related to the increase in intracellular osmotic pressure or biochemical toxicity of cryopreservation because after water efflux from loaded cells, the osmotic pressure and concentration of cryoprotectant inside cells is approximately equal to that outside the cells.

It is believed that influx of penetrating cryoprotectants through the cell membrane during equilibration in vitrification solution containing high concentrations of penetrating cryoprotectants is a main cause of cell damage that occurs during subsequent washing out of the cryoprotectants after cryopreservation.

Kinetics of cryoprotectant permeation inside cells

After the classical work of Kedem, O. et al. (1958) it was generally accepted that the thermodynamic

force responsible for cryoprotectant permeation inside cells is proportional to the cryoprotectant concentration gradient across the cell membrane independent of the composition of the vitrification solution. However, Bronshteyn, V.L. et al. (1994) found that amino acids (glycine and glutamic acid) and carbohydrates (sucrose and sorbitol) significantly diminished ethylene glycol permeation inside *Drosophila melanogaster* embryos. The preventive effect of amino acids was impressive because 1 wt% of glutamic acid + 0.5 wt% glycine practically prevented ethylene glycol permeation inside embryos for up to three hours of equilibration in vitrification solution containing 42 wt% ethylene glycol. The preventive effect of carbohydrates was about four times smaller. These observations show that the approach described in Kedem, O. et al. (1958) and qualitative conclusions obtained based on this model cannot be used to analyze and predict permeation of cryoprotectant inside cells during equilibration in vitrification solution.

Interaction between cryoprotectants and proteins

Timasheff, S.N. (1993) criticized the belief that cryoprotectants form some sort of coating or shell that protects proteins from denaturation during cryopreservation. His criticism was based on the articles of Gekko, K. et al. (1981), Lee, J.C. et al. (1981) and other publications, reporting that cryoprotectants excluded from the surface of proteins. Bronshtein, V.L. (1995b) submitted that the above conclusion of Timasheff and his co-workers is questionable for two reasons. First, the thermodynamic equilibrium in the dialysis experiments of Timasheff and his co-workers cannot be obtained if the hydrostatic pressure inside the dialysis bag is equal to the pressure outside the bag. The suggestion that the effect of this difference in the hydrostatic pressures is negligible is incorrect. Second, amino acids limit penetration of cryoprotectants inside the cell by

decreasing the chemical potential of cryoprotectants in the extracellular aqueous solution (Bronshteyn and Steponkus, 1994). Therefore, cryoprotectant adsorbs at the surface of proteins and partially replaces water molecules hydrating the proteins. The amount of water of hydration, that is, the amount of water at the protein surface that is replaced by molecules of cryoprotectant, increases with increasing concentration of cryoprotectant.

Crowe, J.H. et al. (1990) suggested that freezing and dehydration may be different stress vectors because they found that stabilization of proteins during drying occurs because of an attraction between sugars and proteins. The inventor believes that vitrification of the solution ("shell") at the surface of proteins (and biological membranes) is a general mechanism of protection equally valid for freezing and desiccation.

Effects of intracellular cryoprotectants on the stability of intracellular amorphous state at low temperatures

Steponkus, P.L. et al. (1992) have shown that decreasing osmolarity of the vitrification solution decreases the damaging effect of dehydration in vitrification solution if the dehydration time is several minutes or less. However, to obtain cell survival after cryopreservation, one should successfully vitrify both the extracellular solution and the cytosol. For this reason, Steponkus et al. (1992) suggested that the better cryoprotectant for the loading step is one that allows stable vitrification of cytosol after dehydration in vitrification solution with lower osmolarity. This suggestion was a reflection of a general belief that the presence of cryoprotectants inside cells helps to vitrify cytosol. However, our recent studies (Bronshtein, in preparation) have shown that vitrification temperature of the maximum freeze dehydrated Bovine Serum Aldumin (BSA) solution is T_g=-20°C. In these studies, T_g was estimated as a temperature of detectable onset of ice melting endotherm.

Therefore, T_g in protein solutions is much higher than in solutions of permeating cryoprotectants. This suggests that stability of dehydrated cytoplasm that does not contain cryoprotectants is much higher than that of solutions of permeating cryoprotectants with the same osmotic pressure. This agrees with observations (Steponkus et al., 1992; Langis and Steponkus, 1990) obtained for protoplasts from acclimated rye leaves. They found that the protoplast "loaded with ethylene glycol must be subjected to greater dehydration than those not loaded with ethylene glycol to achieve maximum survival after storage in liquid nitrogen." Bronshteyn and Steponkus (1993) found that intraembryo freezing in non-loaded *Drosophila melanogaster* embryos after dehydration in vitrification solution, occurs at significantly lower temperatures compared to those loaded with 2.125 M ethylene glycol during cooling at 5°C/min. Therefore, contrary to the conventional point of view, addition of low molecular weight cryoprotectants into cytoplasm decreases the stability of the cytoplasm. As such, the present invention is based on scientific theories that are opposite to the prior art described above.

It is, therefore, an object of the present invention to provide a preservation method and a cryoprotectant for cryopreserving cells and multicellular specimens that accounts for the newfound facts that use of low molecular weight cryoprotectants can be detrimental to the cryopreservation process. It is a further object of the present invention to provide a preservation method and a vitrification solution for preserving by vitrification extracellular spaces in the specimen.

SUMMARY OF THE INVENTION

The present invention is directed to a method of preserving cells or multicellular specimens including the step of contacting the specimen with a vitrification solution comprising a permeating cryoprotectant, a non-permeating cryoprotectant and a non-permeating co-solute

that limits the amount of the permeating cryoprotectant that permeates the specimen. The method further includes the step of unloading the specimen by contacting the loaded specimen with a rehydration solution comprising a non-permeating co-solute and, optionally, a permeating cryoprotectant and a non-permeating rehydration cryoprotectant, such that cryoprotectant is removed from the cells of the specimen. Furthermore, the cryoprotectants can be loaded or unloaded in a stepwise manner, in a linear manner, or according to a desired profile.

The present invention is also directed to the vitrification and rehydration solutions for use in connection with the method described above.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to a method for preserving a biological specimen and compositions for achieving the same. Suitable specimens can be single cells (erythocyte, stem cells, sperm, *E. Coli*, yeasts and other cellular microorganisms, etc.) or multicellular tissues (skin, blood vessels, organs, embryos, etc.). The method, vitrification solutions and rehydration solutions described herein minimize toxicity of the vitrification and rehydration solutions and increase intracellular and extracellular vitrification temperatures.

The method includes the step of contacting a specimen or sample with a cryopreservation or vitrification solution. The cryopreservation solution includes a permeating (i.e., low molecular weight) cryoprotectant, a non-permeating (i.e., high molecular weight) cryoprotectant and a non-permeating co-solute that effectively decrease the chemical potential of penetrating cryoprotectants in the vitrification solution. Addition of high molecular weight non-permeating cryoprotectants will increase the vitrification temperature of the cryopreservation solution outside cells. The co-solutes will limit the amount of permeating cryoprotectants that move inside cells and

therefore increase the mass/mass ratio of intracellular protein to permeating cryoprotectant in a dehydrated specimen in cryopreservation solution. This will increase the intracellular vitrification temperature for a given osmotic pressure of cryopreservation solution.

The more co-solutes added, the less cryoprotectant penetrates inside the specimen. The more protein/cryoprotectant ratio inside cells, the higher the intracellular vitrification temperature. However, some minimum amount of cryoprotectant is required inside the cells of the specimen in order to protect the cells against dehydration. For this reason, the concentration of the co-solutes that can be added is limited. The maximum concentration of co-solutes that can be added to cryopreservation solution, to limit penetration of cryoprotectant inside cells, depends upon the minimum amount of cryoprotectant required to protect cells against dehydration in cryopreservation solution. The maximum concentration of co-solutes can be found experimentally for every specific type of permeating cryoprotectants, osmotic pressure of cryopreservation solution, type of co-solute and type of specimen.

As noted above, the invention provides a method for shelf preservation of cells and multicellular specimens at refrigeration or higher temperatures. To increase vitrification temperature outside the cells, cryopreservation solution should contain high molecular weight cryoprotectants, such as dextrans, starches, polyethylene glycol, polyvinylpyrrolidone, Ficol, peptides, etc.

Co-solutes that decrease the chemical potential of penetrating cryoprotectants in aqueous solutions include, but are not limited to:

1. Amino acids: glycine, alanine, glutamic acid, proline, valine, hydroxy-l-proline, beta-aminopropionic acid, aminobutyric acid, beta-ainocaproic acid, aminoisobutyric acid, N-methylglycine, norvaline, and

others that are soluble in water in concentration >0.1 mol/l, and derivatives of amino acids (sarcosine, iminodiacetic acid, hydroxyethyl glycine, etc.) that are soluble in water in concentration >0.1 mol/l.

2. Betaines: betaine and other betaines that are soluble in water in concentration >0.1 mol/l.

3. Carbohydrates: monosaccharide (aldose and ketoses) glyceraldehyde, lyxose, ribose, xylose, galactose, glucose, hexose, mannose, talose, heptose, dihydroxyacetone, pentulose, hexulose, heptulose, octulose, etc., and their derivatives

a. Amino sugars: D-ribose,3-amino-3-deoxy-, chitosamine, fucosamine, etc.;

b. Alditols and inositols: glycerol, erythritol, arabinitol, ribitol, mannitol, iditol, betitol, inositol, etc.;

c. Aldonic, uronic, and aldaric acids that are soluble in water in concentration >0.1 mol/l.; and

d. disaccharides (sucrose, trehalose, etc.).

4. Sugar alcohols (sorbitol, etc.).

To obtain a high intracellular vitrification temperature, the cells should be substantially dehydrated. The dehydration damages the cells due to large repulsive forces between macromolecules inside cells. A small amount of cryoprotectant should be present inside cells in order to decrease these forces. However, the amount of cryoprotectant inside the cells should be kept as low as possible to decrease the toxic effect of the vitrification solution and to increase the intracellular vitrification temperature. All these requirements can be achieved by using cryopreservation solution that contain mixtures of permeating (i.e., low molecular weight) and non-permeating (i.e., high molecular weight) cryoprotectants along with non-permeating co-solutes (amino acids, betaines, sugars, etc. in concentrations from 0.1 - 0.6 mol/l) that effectively decrease the chemical potential of penetrating cryoprotectants in cryopreservation solution.

After dehydration in cryopreservation (vitrification) solution, cells can be stored at a temperature that is lower than the vitrification temperatures both inside and outside the cells of the specimen. Prior to dehydration, cells may be loaded in a low concentration (5-40 wt%), non-damaging solution of permeating cryoprotectant to protect cells from damage during dehydration in cryopreservation solution.

After storage, the samples should be rehydrated and returned to normal physiological medium. In other words, intracellular cryoprotectant should be removed from the cells and exchanged for water. It is believed that damage during rehydration, when cells are transferred from cryopreservation (vitrification) solution to a rehydration (washing) solution, occurs because of an increase in cellular volume beyond initial cellular volumes. To avoid this possibility of damage, one has to include in rehydration solutions, co-solutes, as described above, such as: amino acids, betaines, carbohydrates, or other non-permeating co-solutes that effectively decrease the chemical potential of permeating cryoprotectants in aqueous solutions. The co-solutes are used in concentrations from 0.1 - 0.6 mol/l. Higher co-solute concentrations will more effectively limit the mass of intracellular cryoprotectants, however, when this mass gets very small, the dehydrated cells may be damaged.

The invention allows one to significantly decrease the osmotic pressure of vitrification solution required to obtain a stable vitrification of cells during cooling, to significantly increase extracellular and intracellular vitrification temperatures and the time of cell equilibration (dehydration) in the vitrification solution, without increasing cell damage. This allows one to solve many related problems occurring during equilibration in vitrification solution, storage and rehydration and washing out of intracellular cryoprotectant.

To improve the ability of cells to survive the cryopreservation process described herein, the amounts of permeating cryoprotectant and other components of the cryopreservation solution may be increased in the cryopreservation solution in a stepwise fashion, a linear fashion or according to a desired profile from an initial concentration (≥0%) to an optimal final concentration. The cryopreservation solution and the relative amounts of components thereof may be controlled mechanically or manually. Similarly, to optimize the rehydration process, the contents of the rehydration solution and timing of the rehydration process can be similarly controlled. The optimal initial and final concentrations, as well as the optimum method for increasing the relative concentrations of the components of the cryopreservation and rehydration solutions is determined empirically.

By increasing the intracellular and extracellular vitrification temperatures, one will be able to increase storage temperature up to refrigeration or even room temperature and, therefore, develop method of long-term shelf preservation of cells.

By increasing the equilibration time in vitrification solution, osmotic pressure gradients arising during dehydration of multicellular specimens can be decreased. This is a very important matter because if a portion of cells in the sample is less dehydrated than other portions, it may freeze during subsequent cooling and be damaged.

Limiting the amount of cryoprotectant inside cells simplifies the washing out procedure or completely avoids washing of the intracellular cryoprotectant from cells prior to transfusion or transplantation. This is a very important achievement for blood transfusion, transplantation of embryos and artificial insemination services.

The method of the present invention encompasses dehydration of specimens, cooling samples to a storage

temperature, warming of the samples to ambient temperature, rehydration and washing out of cryoprotectants in rehydration solution, and returning to normal physiological conditions for various medical procedures (transfusions, transplantation, etc.).

The above invention has been described with reference to the preferred embodiment. Obvious modifications and alterations will occur to others upon reading and understanding the preceding detailed description. It is intended that the invention be construed as including all such modifications and alterations insofar as they come within the scope of the appended claims or the equivalents thereof.

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I Claim:

1. A method for preserving a cell or tissue specimen comprising the steps of contacting the specimen with a solution comprising a non-permeating co-solute characterized by its ability to limit the amount of a permeating cryoprotectant to permeate into the specimen.

2. The method for preserving a cell or tissue specimen as claimed in claim 1, wherein the solution further comprises a permeating cryoprotectant and a non-permeating cryoprotectant.

3. The method for preserving a cell or tissue specimen as claimed in claim 1, further comprising the step of contacting the specimen with a cryopreservation solution comprising a permeating cryoprotectant, a non-permeating cryoprotectant and a non-permeating co-solute.

4. The method for preserving a cell or tissue specimen as claimed in claim 2, wherein the cryoprotectant is selected from the group consisting of dimethylsulfoxide, ethylene glycol, propylene glycol and glycerol.

5. The method for preserving a cell or tissue specimen as claimed in claim 2, wherein the non-permeating cryoprotectant is selected from the group consisting of dextrans, starches, polyethylene glycol, polyvinylpyrrolidone, Ficol and peptides.

6. The method for preserving a cell or tissue specimen as claimed in claim 1, wherein the non-permeating co-solute is selected from the group consisting of an amino acid and derivatives thereof, a betaine, a carbohydrate and a sugar alcohol, wherein the carbohydrate is selected from the group consisting of an aldose monosaccharide, a ketose monosaccharide, an amino sugar, an alditol, an inositol,

aidonic, uronic and aldaric acids, disaccharides and polysaccharides.

7. The method for preserving a cell or tissue specimen as claimed in claim 1, wherein the total concentration of non-permeating co-solute in the co-solute solution is between 0.1 and 0.7 mol/l and is equal to a maximum possible concentration that does not substantially damage cells.

8. The method for preserving a cell or tissue specimen as claimed in claim 6, wherein the co-solute is an amino acid.

9. The method for preserving a cell or tissue specimen as claimed in claim 2, wherein the method is performed in two or more stages of contacting the sample with increasingly higher concentrations of the permeating cryoprotectant and the co-solute.

10. The method for preserving a cell or tissue specimen as claimed in claim 2, wherein the method is performed by simultaneously increasing concentrations of both the permeating cryoprotectant and the co-solute from an initial concentration to a final concentration according to a desired profile.

11. The method for preserving a cell or tissue specimen as claimed in claim 2, wherein the rehydration solution further comprises a permeating rehydration cryoprotectant.

12. The method for preserving a cell or tissue specimen as claimed in claim 11, further comprising the step of rehydrating the specimen by contacting the preserved specimen with a rehydration solution comprising a non-permeating rehydration co-solute characterized by its

ability to limit the amount of a permeating cryoprotectant to permeate into the specimen, such that cryoprotectant within the specimen is removed from cells of the specimen.

13. The method for preserving a cell or tissue specimen as claimed in claim 12, wherein the permeating rehydration cryoprotectant is selected from the group consisting of dimethylsulfoxide, ethylene glycol, propylene glycol and glycerol.

14. The method for preserving a cell or tissue specimen as claimed in claim 12, wherein the rehydration step is performed by simultaneously decreasing concentrations of both the permeating rehydration cryoprotectant and the rehydration co-solute from an initial concentration to a final concentration according to a desired profile.

15. The method for preserving a cell or tissue specimen as claimed in claim 12, wherein the non-permeating rehydration co-solute is selected from the group consisting of an amino acid and derivatives thereof, a betaine, a carbohydrate and a sugar alcohol, wherein the carbohydrate is selected from the group consisting of an aldose monosaccharide, a ketose monosaccharide, an amino sugar, an alditol, an inositol, aidonic, uronic and aldaric acids, disaccharides and polysaccharides.

16. The method for preserving a cell or tissue specimen as claimed in claim 1, wherein the contacting step is performed at room temperature or higher.

17. The method for preserving a cell or tissue sample as claimed in claim 1, wherein the specimen can be stably stored at a temperature greater than 4°C.

18. A cryopreservation solution for use in cryopreserving a cell or tissue specimen comprising a permeating cryoprotectant, a non-permeating cryoprotectant and a non-permeating co-solute.

19. The cryopreservation solution as claimed in claim 18, wherein the permeating cryoprotectant is selected from the group consisting of dimethylsulfoxide, ethylene glycol, propylene glycol and glycerol.

20. The cryopreservation solution as claimed in claim 18, wherein the non-permeating cryoprotectant is selected from the group consisting of dextrans, starches, polyethylene glycol, polyvinylpyrrolidone, Ficol and peptides.

21. The cryopreservation solution as claimed in claim 18, wherein the non-permeating co-solute is selected from the group consisting of an amino acid and derivatives thereof a betaine, a carbohydrate and a sugar alcohol, wherein the carbohydrate is selected from the group consisting of an aldose monosaccharide, a ketose monosaccharide, an amino sugar, an alditol, an inositol, aidonic, uronic and aldaric acids, disaccharides and polysaccharides.

22. A rehydration solution for use in rehydrating cryopreserved cell or tissue specimen comprising a permeating rehydration cryoprotectant and a non-permeating rehydration co-solute.

23. The rehydration solution as claimed in claim 22, wherein the permeating rehydration cryoprotectant is selected from the group consisting of dimethylsulfoxide, ethylene glycol, propylene glycol and glycerol.

24. The rehydration solution as claimed in claim 22, wherein the non-permeating rehydration co-solute is selected from the group consisting of an amino acid and derivatives thereof, a betaine, a carbohydrate and a sugar alcohol, wherein the carbohydrate is selected from the group consisting of an aldose monosaccharide, a ketose monosaccharide, an amino sugar, an alditol, an inositol, aidonic, uronic and aldaric acids, disaccharides and polysaccharides.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15611

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A01N 1/02
US CL :435/1.1, 1.2, 1.3; 435/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/1.1, 1.2, 1.3; 435/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 4,980,277 A (JUNNILA) 25 December 1990, column 3, lines 58-69.	1-8, 10, 11, 16, 18-24 ---------- 9, 12-15, 17
X — Y	TITTERINGTON et al. Synthetic and biological macromolecules: protection of mouse embryos during cryopreservation by vitrification. Human Reproduction. 1995, Vol. 10, No. 3, pages 649-653, especially Table III.	1-7, 16, 18-24 ---------- 8-15, 17
X — Y	RALL et al. High *in vitro* and *in vivo* survival of day 3 mouse embryos vitrified or frozen in a non-toxic solution of glycerol and albumin. Journal of Reproduction and Fertility. 1994, Vol. 101, pages 681-688, especially pages 682-683.	1-6, 9, 10, 16, 18-24 ---------- 7, 8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15611

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 5,160,313 A (CARPENTER et al.) 03 November 1992, especially columns 3 and 4.	1-7, 10-16, 18-24 ---------- 8, 9
X --- Y	US 5,217,860 A (FAHY et al.) 08 June 1993, especially column 25.	1, 6, 8, 22-24 ---------- 9-16, 22, 23
X --- Y	US 5,364,756 A (LIVESEY et al.) 15 November 1994, especially examples.	1-7, 9, 11, 12, 14-23 ---------- 8, 10, 13